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CASE REPORT

Novel Mutations in the *PC* Gene in Patients with Type B Pyruvate Carboxylase Deficiency

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Abstract We have investigated seven patients with the type B form of pyruvate carboxylase (PC) deficiency. Mutation analysis revealed eight mutations, all novel. In a patient with exon skipping on cDNA analysis, we identified a homozygous mutation located in a potential branch point sequence, the first possible branch point mutation in *PC*. Two patients were homozygous for missense mutations (with normal protein amounts on western blot analysis), and two patients were homozygous for nonsense mutations. In addition, a duplication of one base pair was found in a patient who also harboured a splice site mutation. Another splice site mutation led to the activation of a cryptic splice site, shown by cDNA analysis.

All patients reported until now with at least one missense mutation have had the milder type A form of PC deficiency. We thus report for the first time two patients with homozygous missense mutations with the severe type B deficiency, clinically indistinguishable from other patients with type B form of PC deficiency.

The mutations found here are novel; it is noteworthy that four Turkish patients did not have any mutations in common, despite the rarity of PC deficiency. There is thus no evidence for recurrent mutations in the Turkish or other populations.

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Pyruvate carboxylase (PC) deficiency (MIM #262150) is a rare autosomal recessive disorder with an incidence of around 1 in 250,000. It is caused by deficiency of the pyruvate carboxylase enzyme, which is encoded by *PC*. PC is a mitochondrial matrix enzyme that converts bicarbonate and pyruvate to oxaloacetate, which is used by phosphoenolpyruvate carboxykinase and by the Krebs cycle. The enzyme has important functions in gluconeogenesis, where it is considered the major regulatory enzyme, and it is also involved in lipogenesis and biosynthesis of neurotransmitters. PC forms a homotetramer with each subunit having one molecule of biotin covalently attached. Mutations in genes involved in biotin metabolism may cause a secondary deficiency that can be treated with biotin supplementation, whereas PC deficiency is not responsive to biotin therapy.

The clinical presentation of pyruvate carboxylase deficiency has been divided into three groups, with some overlap between the groups (Robinson 2001; Marin-Valencia et al. 2010). Group A patients present with lactic acidosis between birth and 5 months of age, and survival up to 5 years. The biochemical parameters are generally

normal, except for elevated alanine and proline. The most prominent symptom is psychomotor retardation. Group B patients have a more severe clinical presentation with neonatal onset of lactic acidosis and neurological symptoms, and death within 3 months. The biochemical parameters are severely abnormal, including elevated lactate/pyruvate and acetoacetate/β-hydroxybutyrate ratios, and elevated citrulline, lysine, proline, alanine and blood ammonia. In both forms, hepatomegaly, seizures and failure to thrive may occur. In addition, a few patients with a mild form of PC deficiency, group C, have been reported.

Here we report the clinical and molecular results, including eight novel mutations, in seven patients with the severe type B form of PC deficiency, referred to our laboratory from 1992 to 2011. Pyruvate carboxylase activity was measured in cultured fibroblasts as described (Hansen and Christensen 1980). DNA and total RNA was extracted by standard methods from cultured fibroblasts with compromised PC activity and the RNA was reverse transcribed by SuperScript II (Invitrogen). The cDNA sequence of PC was PCR amplified (GoTaq PCR kit Promega), purified (ExoZap, Finnzymes) and sequenced (Big Dye Terminator V1,1 (Applied Biosystems)). The genomic sequence of PC was assessed in a similar manner (Primers and conditions are available upon request).

Mitochondria were isolated from cultured fibroblasts as previously reported (Schägger et al. 1994). For electrophoresis, 10 μg of protein was run on a 9 % SDS acrylamide gel, which was blotted onto a PVDF membrane. Mitochondrial biotin–containing proteins (pyruvate carboxylase, the α subunit of 3-methylcrotonyl-CoA carboxylase and the α subunit of propionyl-CoA carboxylase) were detected with HRP-conjugated avidin (Sigma) at a 1:10,000 dilution and Supersignal West Pico substrate (Pierce) (Singh et al. 2005). The blot was exposed to film and developed.

Seven patients were identified with severely decreased PC activity (Table 1). The clinical data are shown in Table 1. All patients had the type B form of PC deficiency with an uneventful pregnancy and birth, and a birth weight in the low to normal range. As in other type B patients, the presenting symptom was most often respiratory distress or lactic acidosis, and eventually all patients developed lactic acidosis, and most patients developed respiratory distress. Additional symptoms previously seen in type B patients were also found in some of the patients reported here: seizures, liver affection, hypo- and hypertonia and dysmorphism. Brain imaging showed bilateral cystic changes in four patients; additional findings were cortical atrophy and leukodystrophy. Plasma citrulline was measured in three patients and found to be elevated in all three. Type B patients usually die within the first 3 months; this was also the case for five of the patients reported here, whereas two of the seven patients survived until 5 months of age.

The mutation analysis revealed eight different mutations, all novel (Table 2). Four of the seven patients were homozygous for *PC* mutations, and three were compound heterozygous.

cDNA analysis of patient 1 showed activation of a cryptic splice site at position c.903+8_9 leading to retention of the first seven nucleotides of intron 8 and thus a change of the correct reading frame and a premature stop codon (Fig. 1a). Analysis of genomic DNA revealed the heterozygous presence of a splice site mutation, c.903+1G>A, at the exon 8 donor site. The cDNA analysis of patient 1 also displayed complete skipping of exon 12. Subsequent genomic analysis of the coding sequence, including ~ 600 bp upstream of exon 12, exon 12 and the entire intron 12 (amplified as one fragment), did not reveal any pathogenic mutations. The patient was, however, heterozygous for a known SNP in intron 11, c.1369-529g>a (rs2077432), arguing against a genomic deletion of exon 12. We therefore suspect that the exon 12 skipping is most likely due to an intronic mutation further upstream in the large intron 11 (intron size: ~11 kb).

In patient 2, only trace amounts of PC cDNA could be generated, but direct genomic sequencing showed compound heterozygous presence of a c.3436dupG mutation, leading to a frameshift, and c.3288+1G>A, expected to compromise correct splicing. Both mutations probably activate the nonsense-mediated mRNA decay (NMD) pathway.

Patients 3 and 4 were homozygous for two different nonsense mutations, c.1240C>T (p.Gln414X) in exon 9 and c.370C>T in exon 3 (p.Arg124X), respectively.

Patient 5 was homozygous for a missense mutation in exon 4, c.615G>C (p.Arg205Ser). p.Arg205 is highly conserved among species (Fig. 1b) and changes a charged arginine to an uncharged serine. It is located in the biotin carboxylation domain of the protein, where two other missense mutations have been found (Monnot et al. 2009).

In patient 6, a homozygous missense mutation in exon 16, c.2606G>A (p.Gly869Asp), was found. The affected amino acid residue is located in the carboxyl transferase domain of the protein, and the mutation changes a highly conserved nonpolar glycine to a polar aspartic acid. The absence of other mutations, the severely decreased activity of PC and the normal amounts of PC protein on western blot makes it highly likely that both p.Arg205Ser and p.Gly869Asp are pathogenic.

In patient 7, cDNA analysis encompassing exons 8 to 14 revealed two abnormal fragments of 684 bp and 897 bp (Fig. 1a), but no product of the expected normal size (829 bp). The 684 bp fragment corresponded to a skipping of exon 12, whereas the 897 bp fragment showed retention of the last 68 bp of intron 11, most likely due to an activation of a cryptic splice acceptor site at position

Table 1 Clinical and laboratory data of seven patients with type B PC deficiency

	1	2	3	4	5	6	7
Gender	M	M	F	F	M	F	M
Consanguinity	–	–	+	+	+	?	+
Ethnic origin	Danish	Somalian	Turkish	Turkish	Turkish	Turkish	Turkish
Pregnancy and birth	N.a.		Uneventful	Polyhydramnios	Uneventful	Ablatio placenta	Uneventful
GA at birth	N.a.	38 weeks	Term	41 weeks	34 weeks	Term	37 weeks
BW	N.a.	3,428 g	2,400 g	2,260 g	2,500 g	2,500 g	2,100 g
Age at onset	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal
Presenting symptom	Seizures	Respiratory distress	Hypotonia	Hypothermia	Respiratory distress	Lactic acidosis	Lactic ketoacidosis
Additional symptoms	Psychomotor retardation	Hypoglycemia, liver affection	Apnoe, hypoxia, hypotonia, seizures	Lactic acidosis, respiratory distress, hypertonia	N.a.	Hypoxia, hypotonia, respiratory distress, facial dysmorphism	Facial dysmorphism, seizures
Brain imaging	N.d.	US: multicystic changes bilaterally	US: minimal ventricular dilatation	US: bilateral cysts	MRI: severe cortical atrophy	MRI: intraventricular retrocerebral subdural hemorrhagia, bilateral periventricular cavitations, brainstem hypoplasia	MRI: leukodystrophy, corpus callosum atrophy, globus pallidus hyperintensity, subependymal cysts
Death age	5 months	2 months	18 days	8 days	21 days	5 months	3 days
PC activity	0.02	0.00	0.15	0.00	0.04	0.00	0.07
Plasma citrulline	N.d.	127	N.d.	240	N.d.	230	N.d.
Lactate	8	22	8	20	7	7.7	10

N.d. Not done, *N.a.* Not available, *US* Ultrasound, *MRI* Magnetic resonance imaging

PC activity was measured in cultured fibroblasts in pkat/mg protein (ref 11.0 ± 3.0). Plasma citrulline was measured in μmol/l (ref < 47). Lactate was the highest plasma lactate measured in mmol/l

Table 2 Mutations in seven patients with PC deficiency

Patient	Nucleotide change	Amino acid change	Mutation type	PC protein on western blot analysis
1	c.903+1G>A/?	Exon skipping/Exon skipping	Splice site/?	Absent
2	c.3288+1G>A/c.3436dupG	Exon skipping/?p.Glu1146GlyfsX26	Splice site/Frameshift	Absent
3	c.1240C>T/c.1240C>T	p.Gln414X/p.Gln414X	Nonsense	Absent
4	c.370C>T/c.370C>T	p.Arg124X/p.Arg124X	Nonsense	Absent
5	c.615G>C/c.61G>C	p.Arg205Ser/p.Arg205Ser	Missense	Normal
6	c.2606G>A/c.2606G>A	p.Gly869Asp/p.Gly869Asp	Missense	Normal
7	c.1369-29a>g/c.1369-29a>g	Intron retention and exon skipping	Branch point	Absent

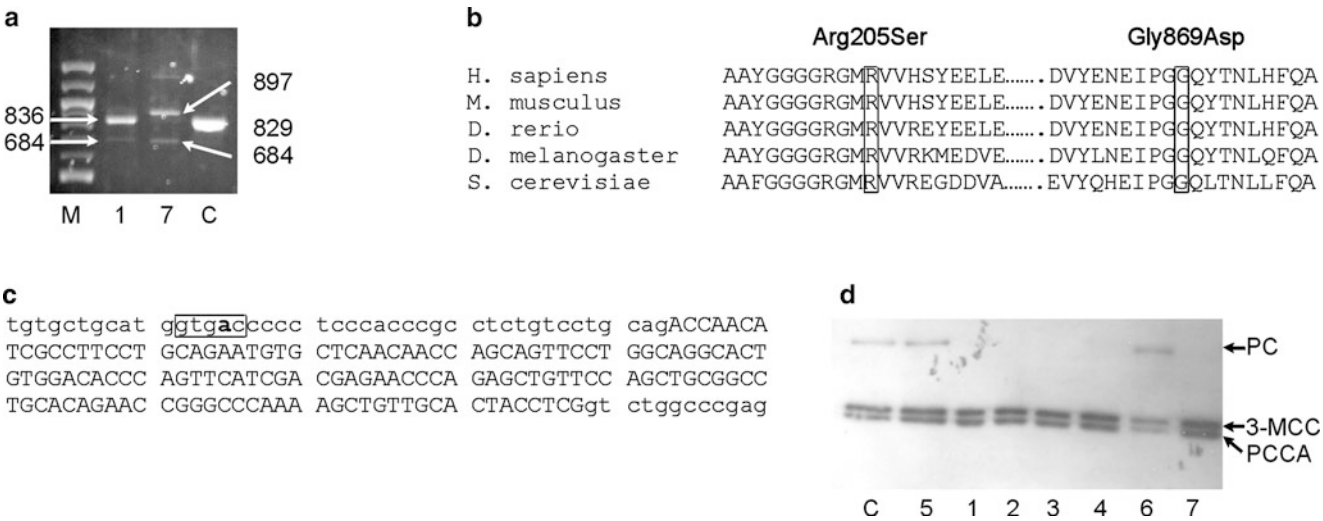

Fig. 1 Analysis of PC protein and sequence analysis. (**a**) cDNA analysis of an exon 8–14 fragment. The normal size fragment of 829 bp is seen in the control (C), whereas a fragment of 684 bp with skipping of exon 12 is seen in patients 1 and 7. Patient 1 also has a band of 836 bp, which corresponds to a fragment with retention of the first 7 nucleotides of intron 8, caused by a splice site mutation, c.903+1G>A. The band of 897 bp in patient 7 represents a fragment with retention of the last 68 bp of intron 11. The intensity of the cDNA fragments in patients 1 and 7 is likely a consequence of activated NMD pathway. (**b**) Alignment of *PC* showing the conservation of the missense mutations found in patients 5 (p.Arg205Ser) and 6 (p.Gly869Asp). (**c**) The genomic sequence of exon 12 and the flanking ~40 bp of intron 11. The c.1369-29A>G mutation (in *bold*) is located in a potential branch point sequence (*boxed*). (**d**) Western blot analysis of protein from fibroblast mitochondria with HRP-conjugated avidin. C: control. 1–7: patients 1–7. The α subunit of 3-methylcrotonyl-CoA carboxylase (3-MCC) and the α subunit of propionyl-CoA carboxylase were used as loading controls

c.1369–69_70. By genomic analysis of exon 12 and ~600 bp of intron 11 we identified a homozygous substitution in intron 11, c.1369-29A>G. The mutation affects a highly conserved adenine in a stretch of five nucleotides (GTG**A**C) with strong resemblance to the consensus branch point sequence Py_{79} T_{75} N A_{92} Py_{75} (79 % of nucleotides are pyrimidine, 75 % thymine, any nucleotide, 92 % adenine, 75 % pyrimidine), except for the first nucleotide, which is a purine (guanine). The possible branch point sequence is located 32–28 bp upstream of the splice acceptor site (consensus 34–21 bp) of exon 12 (Fig. 1c) (Gao et al. 2008).

As expected, western blot analysis showed absence of PC protein in the five patients who had nonsense, frameshift or splicing mutations, whereas normal amounts were found in patients 5 and 6 with missense mutations (Fig. 1d).

This paper adds eight additional mutations to the mutation spectrum in the type B form of PC deficiency. Only a few mutations have been reported previously in patients with the type B form of PC deficiency: four frameshift mutations, an intron retention mutation, a splice site mutation and two missense mutations (Monnot et al. 2009; Carbone et al. 2002; Wexler et al. 1998). All patients reported until now with at least one missense mutation have had the milder type A form of PC deficiency, probably due to an, albeit low, residual activity. We thus report for the first time two patients with homozygous missense mutations with the severe type B deficiency, clinically indistinguishable from the other patients with type B form of PC deficiency.

In addition, we report for the first time a possible branch point mutation in *PC*. The branch point is an element that, together with the polypyrimidine tract, the 5′ and 3′ splice sites and exonic/intronic splicing enhancers/silencers, is essential for correct pre-mRNA splicing, and branch point mutations typically lead to exon skipping, intron retention or the use of a cryptic 3′ splice site, in accordance with our findings of exon skipping and intron retention.

All the mutations found here are novel; it is noteworthy that the four Turkish patients did not have any mutations in common, despite the rarity of PC deficiency. A founder mutation has been found in Canadian Indians (Carbone et al. 1998), but the mutation has not been reported in other populations, where different mutations are found with all families having their private mutation(s). There is thus no evidence for recurrent or founder mutations in other populations, including the Turkish population.

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References

Carbone MA, MacKay N, Ling M, Cole DE, Douglas C, Rigat B, Feigenbaum A, Clarke JT, Haworth JC, Greenberg CR, Seargeant L, Robinson BH (1998 Jun) Amerindian pyruvate carboxylase deficiency is associated with two distinct missense mutations. Am J Hum Genet 62(6):1312–1319

Carbone MA, Applegarth DA, Robinson BH (2002 Jul) Intron retention and frameshift mutations result in severe pyruvate carboxylase deficiency in two male siblings. Hum Mutat 20 (1):48–56

Gao K, Masuda A, Matsuura T, Ohno K (2008 Apr) Human branch point consensus sequence is yUnAy. Nucleic Acids Res 36 (7):2257–2267

Hansen TL, Christensen E (1980) Studies on pyruvate carboxylase from cultured human fibroblasts and amniotic fluid cells. J Inherit Metab Dis 2(2):23–28

Marin-Valencia I, Roe CR, Pascual JM (2010 Sep) Pyruvate carboxylase deficiency: mechanisms, mimics and anaplerosis. Mol Genet Metab 101(1):9–17

Monnot S, Serre V, Chadefaux-Vekemans B, Aupetit J, Romano S, De Lonlay P, Rival JM, Munnich A, Steffann J, Bonnefont JP (2009 May) Structural insights on pathogenic effects of novel mutations causing pyruvate carboxylase deficiency. Hum Mutat 30(5):734–740

Robinson BH (2001) Lactic acidemia: disorders of pyruvate carboxylase and pyruvate dehydrogenase. In: Scriver CR et al (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2275–2296

Schägger H, Cramer WA, von Jagow G (1994 Mar) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal Biochem 217(2):220–230

Singh R, Chénier D, Bériault R, Mailloux R, Hamel RD, Appanna VD (2005 Sep 30) Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate-producing enzymes. J Biochem Biophys Methods 64(3):189–199

Wexler ID, Kerr DS, Du Y, Kaung MM, Stephenson W, Lusk MM, Wappner RS, Higgins JJ (1998) Molecular characterization of pyruvate carboxylase deficiency in two consanguineous families. Pediat Res 43:579–584